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Novel tempeh (fermented soyabean) isoflavones inhibit *in vivo* angiogenesis in the chicken chorioallantoic membrane assay

Serafim Kiriakidis[1]*†‡, Oliver Högemeier[2]‡, Susanne Starcke[1], Frank Dombrowski[2], Jens Claus Hahne[2], Michael Pepper[3], Hem Chandra Jha[1] and Nicolas Wernert[2]

[1]*Institute of Physiological Chemistry and*
[2]*Institute of Pathology, University of Bonn, Bonn, Germany*
[3]*Department of Morphology, University of Geneva Medical Center, Geneva, Switzerland*

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Anti-angiogenic strategies are emerging as an important tool for the treatment of cancer and inflammatory diseases. In the present investigation we isolated several isoflavones from a tempeh (fermented soyabean) extract. The isolated isoflavones were identified as 5,7,4′-trihydroxyisoflavone (genistein), 7,4′-dihydroxyisoflavone (daidzein), 6,7,4′-trihydroxyisoflavone (factor 2), 7,8,4′-trihydroxyisoflavone (7,8,4′-TriOH) and 5,7,3′,4′-tetrahydroxyisoflavone (orobol). The effects on angiogenesis of these isoflavones were evaluated in the chicken chorioallantoic membrane assay; their capacity to inhibit vascular endothelial growth factor-induced endothelial cell proliferation and expression of the Ets 1 transcription factor, known to be implicated in the regulation of new blood vessel formation, were also investigated. We found that all isoflavones inhibited angiogenesis, albeit with different potencies. Compared with negative controls, which slightly inhibited *in vivo* angiogenesis by 6·30 %, genistein reduced angiogensis by 75·09 %, followed by orobol (67·96 %), factor 2 (56·77 %), daidzein (48·98 %) and 7,8,4′-TriOH (24·42 %). These compounds also inhibited endothelial cell proliferation, with orobol causing the greatest inhibition at lower concentrations. The isoflavones also inhibited Ets 1 expression, providing some insight into the molecular mechanisms of their action. Furthermore, the chemical structure of the different isoflavones suggests a structure–activity relationship. Our present findings suggest that the new isoflavones might be added to the list of low molecular mass therapeutic agents for the inhibition of angiogenesis.

Soyabean: Tempeh: Isoflavones: Angiogenesis: Ets 1

Angiogenesis is a conserved process in vertebrates and is essential for many physiological and pathological processes such as development, wound healing, inflammation and tumour vascularization (Folkman, 1995).

Continuing research efforts are directed towards understanding the molecular mechanisms underlying new blood vessel formation. Current models take into account an increasing number of angiogenic and anti-angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin and ephrin families and their receptors, as well as different transcription factors, including *ets 1* (for reviews, see Folkman, 1995; Wernert, 1997; Pepper, 2000; Yancopoulos *et al.* 2000). The roles of many of these factors have been determined by transgenic approaches (Carmeliet & Collen, 2000).

Anti-angiogenic strategies aimed at blocking new blood vessel formation under pathological conditions such as tumour vascularization or rheumatoid arthritis (Wernert *et al.* 2002) are currently emerging. These strategies include: (1) interference with angiogenic ligands, their receptors and downstream signalling; (2) up-regulation of endogenous inhibitors; (3) application of integrin antagonists or inhibitors of matrix-degrading metalloproteinases (Carmeliet & Jain, 2000; Feldman *et al.* 2000; Pfeifer *et al.* 2000; Pepper, 2001).

Successful blockade of angiogenesis has been demonstrated in animal models when monoclonal antibodies or single-chain antibody fragments targeting VEGF (Brekken *et al.* 2000; Vitaliti *et al.* 2000) or the integrin αvβ3 (Brooks *et al.* 1994) were used. The integrin αvβ3 has also been targeted successfully with a cyclic peptide antagonist of integrin αvβ3 (Brooks *et al.* 1994). Other strategies that have been employed to inhibit angiogenesis include intravascular injection of plasminogen-derived angiostatin (Drixler *et al.* 2000), and adenoviral or lentiviral delivery of the extracellular domain of VEGFR-1 (flt-1) (Shiose *et al.* 2000; Takayama *et al.* 2000), endostatin (Chen *et al.* 2000; Feldman *et al.* 2000) or a non-catalytic fragment of matrix metalloproteinase 2 (Pfeifer *et al.* 2000).

Alternative approaches are based on small molecules. Thus, the VEGFR-2 inhibitor SU5416 has been shown to induce a significant reduction of tumour vascular density in an experimental murine brain tumour model (Vajkoczy *et al.* 2000), whereas the fumagillin-derived TNP 470 or AGM 1470 (Ingber *et al.* 1990), one of the first anti-angiogenic compounds described, has already

Abbreviations: CAM, chorioallantoic membrane; 7,8,4′-TriOH, 7,8,4′-trihydroxyisoflavone; VEGF, vascular endothelial growth factor.

* **Corresponding author:** Dr Serafim Kiriakidis, fax +44 20 8383 4499, email s.kiriakidis@imperial.ac.uk
† **Present address:** Renal Section, Division of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK.
‡ S. K. and O. H. contributed equally to the paper.

entered clinical trials (Folkman, 1995; Kerbel, 1997; Belotti & Castronovo, 1998).

Most receptors of angiogenic factors, such as VEGF or basic fibroblast growth factor, as well as many components of receptor-mediated downstream signalling, are tyrosine kinases (Dionne *et al.* 1990; De Vries *et al.* 1992). Tyrosine kinase inhibition is therefore favoured among anti-angiogenic strategies. Genistein is a well-known tyrosine kinase inhibitor (Akiyama *et al.* 1987) that has previously attracted interest as a novel angiogenesis inhibitor (Fotsis, 1995; Fotsis *et al.* 1997). Genistein is an isoflavone (5,7,4′-trihydroxyisoflavone) that can be isolated from tempeh, which is derived from soyabeans and is a popular food in south-east Asian countries.

There is growing experimental evidence from *in vitro* and *in vivo* studies showing that soyabean isoflavones, in particular genistein, function as promising chemopreventive and/or therapeutic agents, inhibiting carcinogenesis and the growth of different human cancers such as breast, prostate and colon cancers (for review, see Sarkar & Li, 2003).

Besides genistein, tempeh contains other isoflavones such as daidzein (7,4′-dihydroxyisoflavone) and 6,7,4′-trihydroxyisoflavone (factor 2; György *et al.* 1964). Two novel isoflavones (7,8,4′-trihydroxyisoflavone (7,8,4′-TriOH) and 5,7,3′,4′-tetrahydroxyisoflavone (orobol)) have been isolated from tempeh in our laboratory in recent years (S Kiriakidis, M Kolvenbach, HC Jha and H Egge, unpublished results). Both are biotransformation products of genistein and daidzein and are produced during the fermentation of soyabeans by enzymes from micro-organisms (Klus & Barz, 1995).

In the present investigation, we tested the *in vivo* anti-angiogenic properties of these novel compounds and of genistein, daidzein and factor 2 by evaluating their effects in the chicken chorioallantoic membrane (CAM) assay. The CAM assay is frequently used for screening purposes due to its economical price, high capacity and ease of manipulation (Auerbach *et al.* 2003).

In order to understand their mechanism of action, we also tested their effect on endothelial cell proliferation and on VEGF-induced Ets 1 expression in cultured endothelial cells, since the Ets 1 transcription factor has been intimately linked to the regulation of new blood vessel formation under both physiological and pathological conditions (Vandenbunder *et al.* 1989; Wernert *et al.* 1992; Iwasaka *et al.* 1996; Valter *et al.* 1999; Wernert *et al.* 1999).

Materials and methods

The isoflavones genistein, daidzein, factor 2, 7,8,4′-TriOH and orobol (for structures, see Fig. 1) were isolated from a methanolic extract of tempeh. Their structures were verified by spectroscopic methods (UV, MS and nuclear magnetic resonance) and by chromatographic comparison (TLC–HPLC) with synthetic substances of known structure. These substances were subsequently synthesized, since the quantity of the isolated substances was not sufficient for the present investigation.

Chemicals, growth factors and antibodies

Chemicals and organic solvents of analytical grade, silica gel 60 F_{254} HP TLC plates and CHOD-PAP reagents were obtained from Merck (Darmstadt, Germany). Sephadex® LH 20 was from Pharmacia (Uppsala, Sweden).

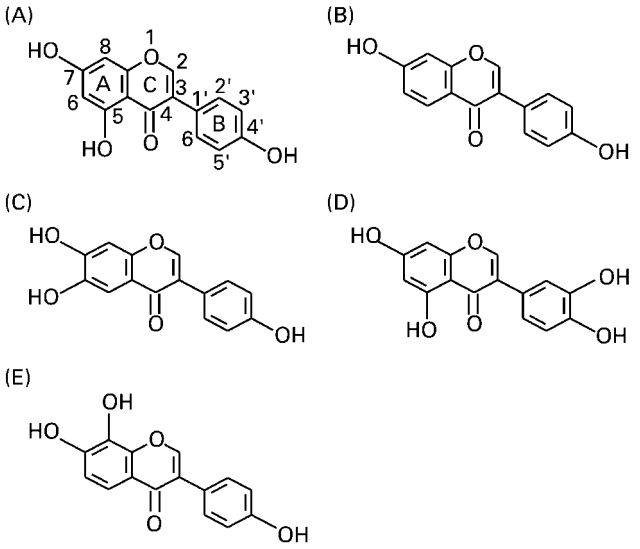

Fig. 1. Chemical structures of tempeh isoflavones. (A), 5,7,4′-trihydroxyisoflavone (genistein); (B), 7,4′-dihydroxyisoflavone (daidzein); (C), 6,7,4′-trihydroxyisoflavone (factor 2); (D), 5,7,3′,4′-tetrahydroxyisoflavone (orobol); (E), 7,8,4′-trihydroxyisoflavone.

Recombinant human VEGF was obtained from TEBU (Frankfurt, Germany). It was stored and used according to manufacturer's recommendations.

For Ets 1 detection, a monoclonal mouse antibody (directed against amino acids 122–288; Transduction Laboratories, Lexington, KY, USA) (Fleischman *et al.* 1995) and a polyclonal rabbit antibody (against amino acids 422–441;, Santa Cruz, CA , USA) (Fisher *et al.* 1992) were used. The α-tubulin antibody was obtained from Santa Cruz Biotechnology.

Isolation of isoflavones from tempeh

Fresh tempeh purchased from a local store (100 g wet weight) was mixed with 300 ml methanol and minced using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) for 5 min. The slurry was filtered through a Buchner filter and the residue treated three times in the same way. Evaporation of the filtrates under vacuum yielded 11·1 g crude tempeh extract. The extract was defatted on a Sephadex LH 20 column (i.d. 20 mm, length 500 mm) using methanol as eluant. Fractions (3 ml) were collected and 5 μl aliquots were spotted onto a silica gel 60 F_{254} HP TLC plate (Merck) followed by development in cyclohexane–dichloromethane–ethylformate–formic acid (35:30:30:5, by vol.). The plates were examined under UV light (λ 254 nm) and the isoflavone spots detected by co-chromatography with authentic isoflavones. After separation of the isoflavones, their structures were determined by spectroscopic methods (UV, MS, nuclear magnetic resonance). The isoflavones eluted from the column shortly after the lipids (e.g. triacylglycerols, phospholipids, NEFA) were completely removed from the column. All isoflavone-containing fractions were pooled and the solvent was evaporated in vacuum (yield 230 mg).

The isoflavone fraction was examined with HPLC using a Chromspher 5B column (3 × 100 mm) and acetonitrile–0·1 M-KH_2PO_4 (35:65, v/v) as eluant at 0·4 ml/min. Detection was at UV 260 nm with recording and integration using Eurochrom 2000 software (Knauer, Berlin, Germany). Genistein and daidzein were present at concentrations of 38 and 18 mg respectively; the

concentrations of the other thee isoflavones ranged between 10 and 5 mg/kg fresh tempeh.

Evaluation of the anti-angiogenic effect of different isoflavones in the chicken chorioallantoic membrane assay

Fertilized chicken eggs were incubated for 2 d at 37°C under a constant relative humidity of 80 %. On day 2, eggs were treated with ethanol (700 g/l). Albumin (3–5 ml) was then aspirated at the egg poles under a laminar flow hood with a sterile needle in order to allow detachment of the developing CAM from the eggshell. Holes were sealed with BIOCLUSIVE™ transparent dressing (Johnson and Johnson, Arlington, TX, USA). Eggs were then incubated in a horizontal position. On day 3 a square-shaped window was cut in the shell using sterile scissors and forceps. This window served as a portal of access for the CAM.

Application of isoflavones on the chorioallantoic membrane assay by the platelet test

Isoflavones were dissolved in ethanol (800 ml/l) to a final concentration of 50 μM. The solution was applied on Thermanox platelets (8 × 8 mm; Nune, Wiesbaden, Germany), which were then dried under a laminar flow hood. Thermanox platelets treated only with ethanol (800 ml/l) served as negative controls.

Platelets with the isoflavone compounds or solution alone were then laid upon the CAM and the eggshells were closed using BIOCLUSIVE™ transparent dressing (Johnson and Johnson). Eggs were returned to the incubator and the results evaluated on day 6 of development.

Computer-assisted densitometric evaluation of angiogenesis

Randomly selected regions were photographed (using a Wild Microscope M420, magnification × 100; Leitz, Oberkochen, Germany) to show blood vessels beneath the platelets. Colour slides were then recorded on a black and white charged-coupled device camera (2720 × 1769 pixel) and digitalized into grey-scale 12 bit. Intensities of clearly visible blood vessel structures varied between 409 and 2694 (range of grey value intensities 0–4096). Blood vessel surfaces were measured by determining the surface of these grey values using the Image Pro Plus Software (Media Cybernetics, Göttingen, Germany).

Cell culture

Human umbilical vein endothelial cells were obtained from TEBU and cultured in Medium 200 (TEBU) with low serum growth supplement in humidified air with 5 % CO_2 at 37°C. After confluence, cells were maintained in medium without low serum growth supplement for 4 h and then stimulated (for 4 h) with VEGF (10 ng/ml) with or without the different tempeh constituents (50 μM). Cells were then processed for protein extraction.

Protein extraction and Western blotting

Cells were lysed in 1 ml TRIzol Reagent (Life Technologies Inc., Carlsbad, CA, USA) and protein was isolated according to the manufacturer's instructions. Equal amounts of protein (20 μg per sample) were separated by SDS–PAGE (1:10, w/v) gel electrophoresis and transferred to a nitrocellulose membrane in buffer containing 25 mM-Tris, 150 mM-glycine and methanol (200 ml/l) at 250 mA for 1 h. Membranes were blocked by incubation for 1 h in blocking solution (10 ml/l) (90 ml Tris-buffered saline (50 mM-Tris, pH 7·5, 150 mM-NaCl) and 10 ml blocking stock solution (100 ml/l; Boehringer Mannheim, Mannheim, Germany)).

Both anti-Ets 1 primary antibodies were diluted 1:1000 in the blocking solution (5 ml/l) and incubated with the membranes overnight at 4°C. Detection was carried out with horseradish (*Armoracia rusticana)* peroxidase-conjugated affinity-purified goat antibody directed against mouse–rabbit IgG (dilution 1:6000 in blocking solution (5 ml/l), 40 min). The reaction was visualized on photographic films by H_2O_2–luminol chemiluminescence detection using a kit (Boehringer Mannheim). Membranes were striped and re-probed with the anti α-tubulin antibody to show equal loading of proteins.

Cell proliferation assay

Endothelial cells were seeded on twenty-four-well plates at 1·5 × 10^4 cells per ml and in Medium 200 (TEBU) with low serum growth supplement in humidified air with 5 % CO_2 at 37°C and incubated overnight to reach optimal cell adhesion. After adhesion, recombinant human VEGF (10 ng/ml) alone or together with the tempeh isoflavones at concentrations of 6, 12, 25 or 50 μM (from a stock solution in dimethylsulfoxide) were added to the culture dishes. Dimethylsulfoxide was used as a vehicle control. The isoflavones or dimethylsulfoxide only were added 1 h before VEGF on the first day of treatment. Cells were then incubated for another 3 d and were counted with a haemocytometer after Trypan Blue staining for viability testing.

Statistical analysis

All data presented are from a representative experiment and the total number of experiments performed is indicated. Statistical analyses on the computer-assisted densitometric evaluation of angiogenesis were carried out using the 'One sample – Student's *t* test' and the Duncan's test for multiple comparisons using SPSS software (SPSS Inc., Chicago, IL, USA). Comparison between different eggs within the single isoflavone groups and control groups was performed using the 'One sample – Student's *t* test' considering the same variances. In addition, multiple comparisons between the different isoflavones were made by use of Duncan's testing using the SPSS software.

Results

Tempeh isoflavones inhibit angiogenesis in the chicken CAM. Densitometric evaluation of blood vessel surfaces revealed *in vivo* anti-angiogenic effects of all isoflavones examined. Results are given in Fig. 2. Compared with negative controls that slightly inhibited *in vivo* angiogenesis by 6·30 %, genistein reduced angiogenesis by 75·09 %, followed by orobol (67·96 %), factor 2 (56·77 %), daidzein (48·98 %) and 7,8,4′-TriOH (24·42 %) (Table 1). With the Duncan's test we found no significant differences between the average reduction of blood vessels observed between genistein and orobol (P=0·323). In addition there was no significant difference found between orobol and factor 2 (P=0·217). Finally, there was no significant difference between factor 2 and daidzein (P=0·343).

Inhibition of vascular endothelial growth factor-stimulated endothelial cell proliferation by tempeh isoflavones

In order to analyse possible mechanisms by which tempeh isoflavones might inhibit angiogenesis in the CAM, we next assessed their effects on VEGF-stimulated human umbilical vein endothelial cell proliferation. As shown in Fig. 3, we found that genistein and orobol exerted the strongest anti-proliferative effects, with orobol showing the highest inhibition at lower concentration (10 μM). Strong effects were also found for the other compounds at a concentration of 50 μM.

Tempeh isoflavones inhibit vascular endothelial growth factor-induced Ets 1 expression in cultured endothelial cells

We then wished to elucidate possible molecular mechanisms by which tempeh isoflavones might inhibit angiogenesis. To this end, we investigated their effects on VEGF-induced expression of the Ets 1 transcription factor, which has been strongly related to angiogenesis under both physiological and pathological conditions.

By Western blot analysis we found a strong induction of both the p51 and the p39 Ets 1 proteins 4 h after VEGF stimulation of human umbilical vein endothelial cells. These two proteins are the translation products of the full-length Ets 1 transcript (p51) and a splice variant lacking exon VII (p39; Fisher *et al.* 1992). As shown in Fig. 4, VEGF-induced expression of p51 was inhibited by genistein and by orobol, whereas the other isoflavones (factor 2, daidzein and 7,8,4′-TriOH) had no effect. Expression of p39 was strongly reduced by all isoflavones.

Discussion

Angiogenesis associated with solid tumours is believed to promote the proliferation, invasion and metastasis of cancer cells. Indeed, multiple genes, including VEGF, involved in angiogen-

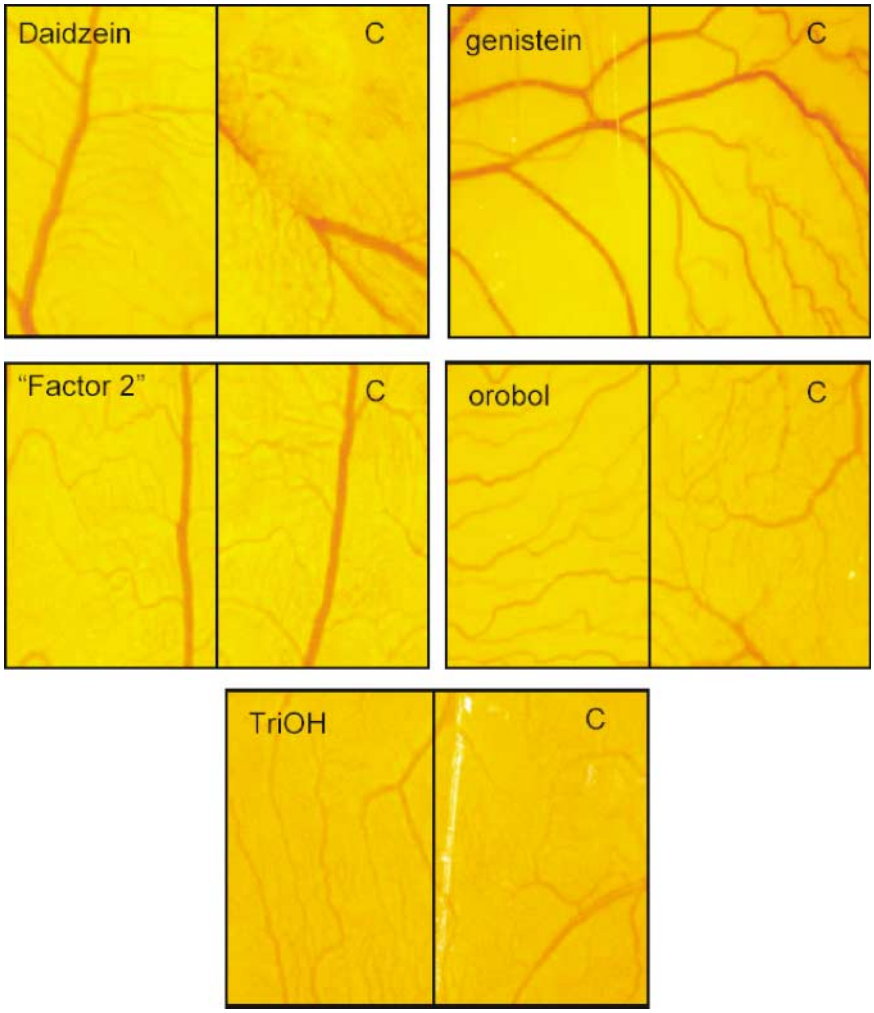

Fig. 2. Examples of inhibitory effects of different isoflavones on *in vivo* angiogenesis in the chicken chorioallantoic membrane assay. (A), daidzein; (B), genistein; (C), factor 2; (D), orobol; (E), 7,8,4′-trihydroxyflavone. Seven to ten eggs per isoflavone (one platelet per egg) were used to determine anti-angiogenic effects. Six to ten randomly selected regions were photographed (Wild Microscope M420, magnification × 100;) to show blood vessels beneath the platelets and in a neighbouring control (C) area without platelets. Platelets without isoflavones served as a negative control. Chorioallantoic blood vessels are visible beneath the transparent platelets. Note in control (C) the presence of an increased number of blood vessels. Conversely, in the region treated with the effective isoflavones genistein and orobol, only a very few new blood vessels can be seen. For details of procedures, see p. 319 and for details of the chemical structures of the isoflavones, see Fig. 1.

Table 1. Inhibitory effects of different isoflavones on angiogenesis in the chicken chorioallantoic membrane assay: comparison of reduction of blood vessel surfaces before and after normalization*

(Mean values and standard deviations)

	Isoflavone test groups (plus platelets)†											
	Control		Genistein		Orobol		Factor 2		Daidzein		7,8,4′-TriOH	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Reduction in blood vessel surface (%)	6·35	3·16	81·44	11·94	73·31	18·33	63·12	25·79	55·33	17·77	30·77	14·41
t value	5·64		21·57		12·00		8·82		9·34		5·64	
Statistical significance of effect (*P* value)	0·0008		<0·0001		<0·0001		<0·0001		<0·0001		0·0013	
Normalized reduction of blood vessel surface and normalized standard failure (%)	0		75·09	3·94	67·96	6·21	56·77	7·24	48·98	6·03	24·42	5·22

7,8,4′-TriOH, 7,8,4′-trihydroxyisoflavone.

* For details of procedures, see p. 319, and for details of chemical structures of isoflavones, see Table 1.

† Test group sizes (number of eggs): control 8, genistein 10, orbol 9, factor 2 13, daidzein 9, 7,8,4′-TriOH 7.

esis have also been implicated in cancer progression and metastasis. Thus, angiogenesis blockade is currently emerging as a novel approach of great potential for cancer treatment, and continuous efforts are made to develop new anti-angiogenic strategies.

Several approaches are based on small molecules such as the VEGFR-2 inhibitor SU5416, which has been shown to induce a significant reduction of tumour vascular density in an experimental murine brain tumour model (Vajkoczy *et al.* 2000). One of the first anti-angiogenic compounds, the fumagillin-derived TNP 470 or AGM 1470 (Ingber *et al.* 1990), has already entered clinical trials (Folkman, 1995; Kerbel, 1997; Belotti & Castronovo, 1998).

The isoflavone genistein, a major ingredient of soyabean, has also been found to inhibit angiogenesis *in vitro* and *in vivo* and

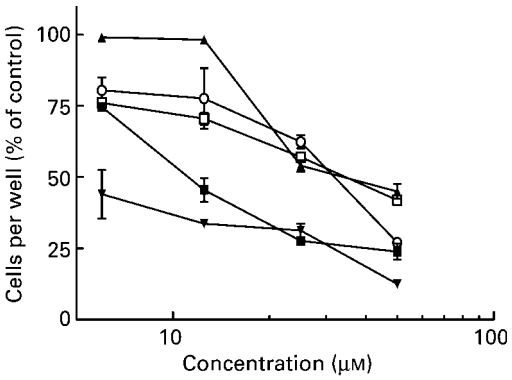

Fig. 3. Effect of increasing concentrations of tempeh isoflavones on the proliferation of human umbilical vein endothelial cells. Cells were seeded at a density of $1{\cdot}5 \times 10^4$ cells per well in twenty-four-well plates. After 24 h cells were pre incubated with the tempeh isoflavones genistein (■), daidzein (▲), factor 2 (□), orobol (▼), 7,8,4′-trihydroxyflavone (○) at concentrations of 0–50 μM or with vehicle (dimethylsulfoxide) for 1 h. Cells were then stimulated with 10 ng vascular endothelial growth factor/ml cultured for a further 3 d and then counted using a haemocytometer. For details of procedures, see p. 319 and for details of the chemical structures of the isoflavones, see Fig. 1. Values are expressed as a percentage of controls, i.e. dimethylsulfoxide treated–vascular endothelial growth factor stimulated cells only (usually in the range of 80 000 cells per well). Values are means for triplicate wells with standard errors shown by vertical bars. A representative of six independent experiments is shown.

tumour cell proliferation and tumour growth *in vivo*. In addition, soyabean isoflavones have been identified as dietary components that have an important role in reducing the incidence of different human cancers, such as those of the breast and prostate (for review, see Sarkar & Li, 2003).

In the present study, we have described the isolation of novel isoflavones from tempeh, a fermented soyabean product and a very popular food in south-east Asian countries. Using biological tests we found that the novel isoflavones exhibited anti-angiogenic effects *in vivo* in the chicken CAM assay and inhibited VEGF-induced endothelial cell proliferation *in vitro* in a dose-dependent manner. Although the relative concentration of orobol in tempeh was lower in comparison with the concentration of genistein, we found that both isoflavones had similar anti-angiogenetic potency. It should be noted that in VEGF-stimulated endothelial cells, orobol had a stronger anti-proliferative effect than genistein at low concentrations (<20 μM).

In order to identify possible molecular mechanisms by which isoflavones might exert their anti-angiogenic effect, we investigated their ability to inhibit VEGF-induced expression of the Ets 1 transcription factor, which has been intimately linked to the regulation of angiogenesis in endothelial cells. Previously, we have shown that antisense oligodesoxynucleotide blockade of Ets 1 inhibits angiogenesis in the chicken CAM assay (Wernert *et al.* 1999) and that the anti-angiogenic compound fumagillin strongly decreases VEGF-induced Ets 1 expression in cultured endothelial cells (Wernert *et al.* 1999). In the present study, we used both a monoclonal mouse antibody, which recognizes p51 (the full-length isoform of Ets1) and a polyclonal rabbit antibody, which can also recognize products of alternatively spliced mRNA products such as p39 (p39 lacks exon VII) (Jorcyk *et al.* 1991; Kilpatrick *et al.* 1999). We found that several isoflavones inhibited Ets 1 expression, which suggests that Ets 1 inhibition might be one of the molecular mechanisms underlying anti-angiogenesis in these settings. Inhibition involved p51 Ets 1 and p39 Ets 1; the latter is translated from a splice variant lacking exon VII. This exon encodes part of the DNA-binding domain of Ets 1, other parts being encoded by exons VIII and IX (Jorcyk *et al.* 1991). Whether p39 displays DNA-binding properties other than p51 Ets 1 remains to be determined. In colon carcinoma cells p39 Ets 1 has been shown to

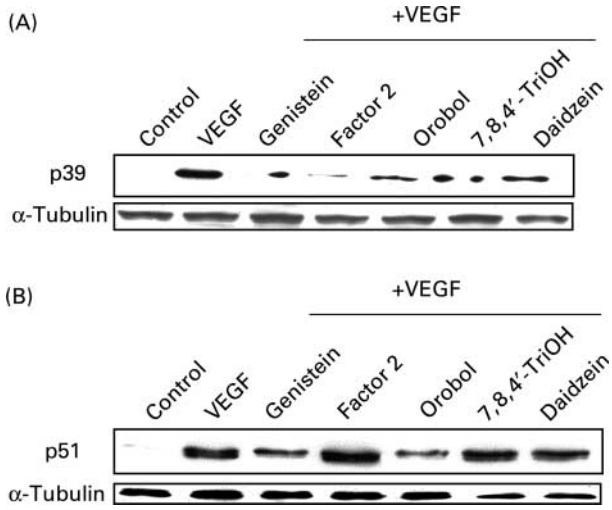

Fig. 4. Effects of different tempeh isoflavones on vascular endothelial growth factor (VEGF)-induced expression of p51 Ets 1 (A) and of p39 Ets 1 (B) in cultured human umbilical vein endothelial (HUVE) cells. 7,8,4′-TriOH, 7,8,4′-trihydroxyflavone. HUVE cells were cultured in Medium 200 (TEBU, Frankfurt, Germany) with a low serum growth supplement (LSGS; TEBU). Confluent cells were LSGS-starved for 4 h and then stimulated for 4 h with VEGF (10 ng/ml) with or without tempeh isoflavones (50 μM). Proteins were extracted with TRIzol reagent, separated by SDS–PAGE, followed by electrotransfer onto nitrocellulose membranes. For the detection of Ets1 proteins, a polyclonal rabbit antibody (directed against amino acids 422–441, (A)) and a monoclonal mouse antibody (against amino acids 122–288 (B)) were used. Membranes were re-probed using an anti-α-tubulin antibody to show equal protein loading. For details of procedures, see p. 319 and for details of the chemical structures of the isoflavones, see Fig. 1. A representative of three independent experiments is shown. VEGF strongly induced both p51 (A) and p39 Ets 1 proteins (B). Induction of p51 Ets 1 was moderately inhibited by genistein and by orobol, whereas the other isoflavones had no effect (A). Expression of p39 Ets 1 was strongly inhibited by all isoflavones (B).

rescue Fas-induced apoptosis (Huang *et al.* 1997; Li *et al.* 1999; Shiose *et al.* 2000).

The finding that the two splice variants of Ets 1 are differentially influenced by the isoflavones could be a hint of different mechanisms of inhibition that they cause. We found that factor 2, 7,8,4′-TriOH and daidzein only reduced the expression of the p39 Ets 1 splice variant. Although this could be achieved by a general inhibitory effect on the spliceosome, this is unlikely, as both genistein and orobol were able to reduce the Ets 1 expression rate of p39 as well as of p51. Therefore, it appears that genistein and orobol are specific inhibitors of the VEGF-induced expression of Ets 1. This finding fits well with the observation that genistein and orobol are the most potent inhibitors of angiogenesis in the CAM assay and that they exert the strongest anti-proliferative effects in the cell proliferation assay.

Comparison of the chemical structure of the different isoflavones suggests potential structure–activity relationships (Fig. 1). The 4′-OH group present in ring B of genistein (and all other compounds) has been previously shown to be essential for the tyrosine kinase inhibitory activity and the anti-tumour properties of genistein (Fotsis *et al.* 1997). Moreover, isoflavones possess a polyphenol structure, and it is known that *o*-dihydroxy groups in phenols enable them to function as strong antioxidants, capturing free oxygen radicals implicated in signal transduction pathways, which leads to cell proliferation (Fotsis *et al.* 1997). *o*-Dihydroxy groups are present at 3′,4′ of ring B in orobol, which thus possesses both elements supposed to be necessary for anti-proliferative effects (a 4′-OH group and an *o*-dihydroxy-group).

By evaluating the antioxidant activity of the isoflavones using the method of superoxide dismutase-inhibitable O_2^- production by xanthine–xanthine oxidase, we found that the antioxidant activity of orobol was greater than that of genistein. Furthermore, a mixture of the isoflavones showed much higher inhibition of O_2^- production compared with individual isoflavones, probably due to a synergistic effect (S Kiriakidis, M Kolvenbach, HC Jha and H Egge, unpublished results).

It has been reported recently that reactive oxygen species, which induce migration and proliferation of cultured endothelial cells, also induce Ets 1 expression (Yasuda *et al.* 1999). Inhibition of both Ets 1 expression and endothelial cell proliferation by isoflavones could therefore be due to their tyrosine kinase inhibitory as well as their antioxidative effects. There is no *o*-dihydroxy group in daidzein, which is also the least hydroxylated of the investigated compounds. This may explain its low activity in some of our present experiments.

Although genistein is likely to be the major active component in tempeh, and the majority of the studies so far refer to genistein and daidzein as the sole soyabean isoflavones, our present results show, for the first time to our knowledge, that other active isoflavones are also present in tempeh. These isoflavones might become additional low molecular mass compounds that have potential for the inhibition of angiogenesis and angiogenesis-related diseases.

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